Journal of **R**eproduction & **I**nfertility
Volume 27, Issue no. 2
https://doi.org/10.18502/jri.v27i2.23150

Original Article

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Free Fatty Acid Analysis of Plasma from Different Stages of Endometriosis: A Preliminary Study

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Received: 9, Oct. 2026
Accepted: 25, Oct. 2026

Abstract

Background: Endometriosis is a chronic, estrogen-dependent inflammatory disease associated with pelvic pain, dysmenorrhea, dyspareunia, and infertility. This study aimed to characterize serum free fatty acid (FFA) profiles in women with histopathologically confirmed mild and severe endometriosis and explore selected FFAs and FFA ratios as potential markers of disease severity.

Methods: This observational study included 20 women with histopathologically confirmed endometriosis, classified as mild (stages I–II; n=10) or severe (stages III–IV; n=10). Serum FFAs were extracted using the Folch method and analyzed by flame ionization detection (FID). Data were normalized and analyzed using MetaboAnalyst. Univariate and receiver operating characteristic (ROC) analyses were performed to identify potential biomarkers distinguishing disease severity. Statistical significance was set at $p<0.05$.

Results: The identified FFAs included lauric acid (C12:0), capric acid (C10:0), myristic acid (C14:0), stearic acid (C18:0), linoleic acid (C18:2), and dihomo-γ-linolenic acid (DGLA; C20:3). Although relative increases and decreases were observed, individual FFA levels did not differ significantly between the groups. The lauric acid/myristic acid ratio differed significantly between groups ($p=0.04$; AUC= 0.77), whereas the lauric acid/linoleic acid ratio showed a borderline difference ($p= 0.05$; AUC=0.75).

Conclusion: The lauric acid/myristic acid ratio showed preliminary potential for distinguishing mild from severe endometriosis in women with an established diagnosis. These findings do not establish its utility for primary screening or diagnosis. Larger studies with appropriate control groups, standardized sample collection, and independent validation are needed before clinical application can be considered.

Keywords: Biomarkers, Disease severity, Endometriosis, Free fatty acid.
To cite this article: Bigdeli Gh, Mirzaei N, Shakeri M, Ghobeh M, Padmehr R, Zafardoust S, et al. Free Fatty Acid Analysis of Plasma from Different Stages of Endometriosis: A Preliminary Study. J Reprod Infertil. 2026;27(2):152-156. https://doi.org/10.18502/jri.v27i2.23150.

Introduction

Endometriosis is a chronic, estrogen-dependent inflammatory disease characterized by the presence of endometrial-like tissue outside the uterine cavity. It affects approximately 10% of women of reproductive age and is associated with dysmenorrhea, chronic pelvic pain, dyspareunia, reduced quality of life, and infertility. Although clinical assessment and imaging may support evaluation, definitive diagnosis and disease staging have historically relied on laparo-

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scopic assessment and, where indicated, histopathological confirmation. There remains interest in identifying non-invasive approaches that may help characterize disease severity; however, biomarkers intended for severity discrimination must be distinguished from tests intended to diagnose endometriosis in individuals without an established diagnosis (1-4).

Dietary fats are not merely an energy source; their fatty acid composition actively modulates inflammatory pathways implicated in endometriosis. *In vitro*, endometrial cells from women with and without endometriosis respond differently to the fatty acid profile of the culture medium, indicating that specific fatty acids can alter cell survival and behavior. Consistent with this, Bedaiwy et al. reported that exposure to particular fatty acids increased inflammatory marker levels (1). By contrast, a high-antioxidant diet has been shown to attenuate oxidative stress and slow endometriosis progression (2).

Despite these observations, comprehensive analyses of serum free fatty acid (FFA) profiles across endometriosis severity groups remain limited. The present study did not evaluate primary diagnostic performance because it included no healthy or disease-free control group. Instead, it compared women with histopathologically confirmed mild and severe endometriosis. Therefore, the purpose of the current study was to characterize serum FFA profiles in these two groups and to explore whether selected FFAs or FFA ratios may help discriminate disease severity. The findings are exploratory and are not intended to establish a screening or diagnostic test for endometriosis.

Methods

Study design and participants: This observational study was conducted at Avicenna Fertility Clinic, affiliated with Avicenna Research Institute (ARI). The study protocol was approved by the ARI Ethics Committee (IR.ACECR.Avicenna.REC.1399.007), and written informed consent was obtained from all participants before enrollment.

The stage of disease was confirmed by the gynecologist and the conclusive diagnosis was verified through a histopathological report. The classification was based on ESHRE guidelines (3). Patients with stage I or II disease were categorized as having mild endometriosis, whereas those with stage III or IV disease were classified as having severe endometriosis.

A total of 20 women aged 20–40 years who underwent diagnostic or therapeutic laparoscopy were included in the study. Participants comprised 10 women with mild endometriosis (stages I–II) and 10 women with severe endometriosis (stages III–IV).

The exclusion criteria included pregnancy, lactation, hormonal therapy within the previous 3 months, malignancy, chronic inflammatory diseases, and metabolic disorders, including diabetes mellitus and dyslipidemia. Blood samples were collected following a 12-*hr* overnight fast for serum FFA analysis.

Serum FFA analysis: Approximately 500 μl of venous blood was collected from each participant. Blood samples were allowed to clot and then centrifuged at 13000 *rpm* for 1 *min* to separate the serum. The serum samples were subsequently transferred into labeled tubes and stored at $-20°C$ until analyze.

For serum FFA profiling, 50 μl of thawed serum was subjected to lipid extraction and derivatization prior to gas chromatographic analysis and FFA extraction was performed according to the Folch method using a chloroform/methanol mixture (2:1, *v/v*). Briefly, 100 μl of the extraction solvent was added to 50 μl of serum, followed by vortex mixing and centrifugation at 4000 *rpm* for 15 *min*. The organic phase containing the extracted fatty acids was carefully collected and evaporated to dryness.

The dried lipid residue was subsequently derivatized to fatty acid methyl esters (FAMEs) using a two-step transesterification procedure. First, potassium hydroxide (KOH) was used, followed by boron trifluoride (BF_3) in methanol. This procedure converted the fatty acids into their corresponding methyl ester derivatives suitable for gas chromatographic analysis.

FAME identification was performed by comparison with authentic FAME standards (GLC-462; Nuchek Prep, USA). Chromatographic analysis was performed using an Agilent 7890A gas chromatograph (Agilent Technologies, USA) equipped with a G4513A automatic liquid sampler and a flame-ionization detector (FID) (5).

Separation was achieved using a CP-Sil 88 capillary column (100 m×0.25 *mm* internal diameter, 0.20 μm film thickness; Agilent Technologies, USA). A 1-μl aliquot of the derivatized sample was injected into the GC system. The area percentage of each FAME peak was calculated, and

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individual fatty acids were identified based on their retention times relative to those of the corresponding authentic standards. The results were expressed as weight percentages (wt%) of individual fatty acids relative to the total fatty acid content detected in the serum sample.

Statistical analysis: Descriptive analyses were performed to characterize the study population and summarize the serum FFA profiles. Continuous variables were expressed as mean±standard error of the mean (SEM). Prior to statistical analysis, FFA data was normalized. The distribution of the data was assessed using the Kolmogorov–Smirnov test. Statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad, USA). A two-sided p-value of <0.05 was considered statistically significant. Furthermore, biomarker data analysis was performed using MetaboAnalyst version 6.0.

Results

Univariate analysis of serum FFAs: Serum FFA profiles from patients with mild endometriosis (stage I–II) and severe endometriosis (stage III–IV) were analyzed by gas chromatography coupled with FID. Prior to statistical analysis, the data was normalized. Univariate analysis was subsequently performed using the MetaboAnalyst platform to compare serum FFA profiles between patients with mild and severe endometriosis.

The identified serum FFAs were lauric acid (C12:0), capric acid (C10:0), myristic acid (C14:0), stearic acid (C18:0), linoleic acid (C18:2), and dihomo-γ-linolenic acid (DGLA; C20:3). Relative increases and decreases were observed between the two groups; however, the individual fatty acids were not show a statistically significant difference (p<0.05) (data not shown).

Biomarker analysis: Following log transformation and normalization of the FID-derived serum fatty acid data, biomarker analysis was performed using the MetaboAnalyst platform. The ratio of lauric acid to myristic acid and the ratio of lauric acid to linoleic acid were identified as potential discriminatory biomarkers between patients with mild and severe endometriosis (Table 1).

Unlike the individual fatty acid measurements, the lauric acid-to-myristic acid ratio showed a statistically significant difference between the two groups based on an independent-samples t-test (p=0.04), whereas the lauric acid-to-linoleic acid ratio showed a borderline statistically significant difference (p=0.05). These findings suggest that the relative balance between lauric acid and myristic acid, as well as between lauric acid and linoleic acid, may provide greater discriminatory information between mild and severe endometriosis than individual serum FFA levels alone.

Table 1. Potential serum FFA ratio biomarkers distinguishing mild and severe endometriosis

Biomarkers	AUC	p-value
Lauric acid/myristic acid	0.77	0.04
Lauric acid/linoleic acid	0.75	0.05

The serum lauric acid-to-myristic acid ratio was further evaluated as a potential biomarker for discriminating between mild and severe endometriosis. As illustrated in figure 1, the lauric acid/myristic acid ratio was lower in patients with mild endometriosis and higher in those with severe endometriosis. Thus, this ratio showed a relative decrease in mild endometriosis and a relative increase in severe endometriosis.

The difference in the lauric acid/myristic acid ratio between the two groups was statistically significant (p=0.04). These findings suggest that the serum lauric acid-to-myristic acid ratio may serve as a candidate biomarker for differentiating mild from severe endometriosis. To further evaluate the discriminatory ability of the lauric acid-to-myri-

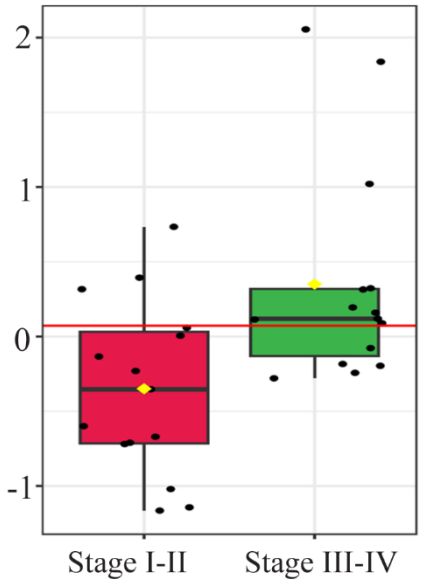


Figure 1. Boxplot of the serum lauric acid-to-myristic acid ratio in mild and severe endometriosis. The lauric acid/myristic acid ratio was significantly lower in patients with stage I–II endometriosis than in those with stage III–IV endometriosis

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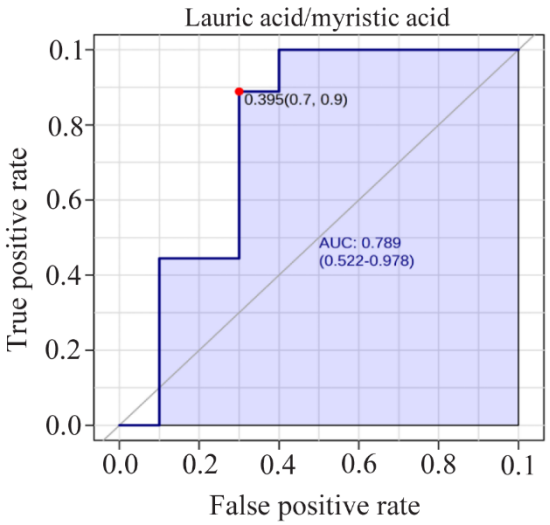


Figure 2. ROC curve of the serum lauric acid-to-myristic acid ratio for discriminating between mild (stage I–II) and severe (stage III–IV) endometriosis (AUC = 0.77)

stic acid ratio between mild and severe endometriosis, receiver operating characteristic (ROC) curve analysis was performed (Figure 2).

Discussion

In this study, serum FFA profiles were compared between women with histopathologically confirmed mild endometriosis (stage I–II) and severe endometriosis (stage III–IV). Although none of the individual fatty acids differed significantly between the two groups, relative changes were observed. Capric acid (C10:0), myristic acid (C14:0), stearic acid (C18:0), linoleic acid (C18:2 n-6), and dihomo-γ-linolenic acid (DGLA; C20:3 n-6) showed lower relative values in stage III–IV than in stage I–II, whereas lauric acid (C12:0) showed a higher relative value. These relative changes should not be interpreted as statistically significant differences because the individual fatty acids did not differ significantly between groups. The lauric acid/myristic acid and lauric acid/linoleic acid ratios were identified as candidate markers of severity discrimination, with the lauric acid/myristic acid ratio showing a statistically significant between-group difference.

Endometriosis is a chronic gynecological condition characterized by the presence of endometrial-like tissue, including glands and stroma, outside the uterine cavity. It is recognized as an estrogen-dependent inflammatory disorder (4). Fatty acids have important structural and metabolic functions and also participate in the regulation of inflammatory responses through their conversion into bioactive lipid mediators (6). Consequently, alterations in circulating fatty acid composition may reflect changes in lipid metabolism and inflammatory processes associated with endometriosis.

The lower relative level of stearic acid (C18:0) observed in patients with severe endometriosis is of particular interest. Previous research has reported alterations in serum fatty acid composition in women with endometriosis. Khanaki et al. found that stearic acid (C18:0) levels were significantly lower in women with endometriosis than in control participants (7). Although their study did not directly compare stage I–II with stage III–IV disease, this finding is consistent with the direction of change observed for stearic acid (C18:0) in the present study. However, because the difference in stearic acid (C18:0) between the two disease-severity groups was not statistically significant in our study, this finding should not be interpreted as evidence that stearic acid (C18:0) is an independent marker of severe endometriosis.

The observed reduction in linoleic acid (C18:2 n-6) and dihomo-γ-linolenic acid (DGLA; C20:3 n-6) in severe endometriosis may also be relevant to the inflammatory environment associated with the disease. Polyunsaturated fatty acids (PUFAs) are involved in the synthesis of lipid mediators that regulate inflammatory responses (5). Previous studies have reported an association between higher dietary intake of PUFAs and lower levels of inflammation (8). Nevertheless, circulating fatty acid profiles may be influenced by dietary intake, fasting duration, menstrual cycle phase, metabolic status, and other patient-related factors. The availability of these covariates in the present study should therefore be considered when interpreting the results.

The lauric acid/myristic acid ratio was higher in stage III–IV than in stage I–II disease (p=0.04; AUC=0.77). The lauric acid/linoleic acid ratio showed a between-group difference of borderline significance (p=0.05; AUC=0.75). These findings suggest that relative relationships between fatty acids may provide information not apparent from individual FFA measurements. However, the AUC values indicate only moderate discriminatory performance, and the results are exploratory.

Crucially, this study included only 20 women with confirmed endometriosis and did not include healthy or disease-free controls. Consequently, the ratios assessed in the present study are not suitable for use as screening or diagnostic biomarkers,

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whether for identifying endometriosis within the general population or for discriminating between affected women and healthy individuals. At most, the results support further investigation of these ratios as candidate markers for stratifying surgical disease severity among patients who already have an established diagnosis. The small sample size, potential unmeasured confounding, and need for independent validation further limit clinical interpretation.

Conclusion

The present study evaluated differences in disease severity among women with histopathologically confirmed endometriosis and did not include a healthy control group. The lauric acid (C12:0)/ myristic acid (C14:0) ratio showed the potential for discriminating between mild (stage I–II) and severe (stage III–IV) endometriosis, suggesting its possible utility as a candidate biomarker for stratifying surgical disease severity. However, its clinical applicability should be interpreted cautiously. These findings cannot be extrapolated to screening for or diagnosing endometriosis in the general population, not to distinguishing affected individuals from healthy individuals. Furthermore, the observed discriminatory performance (AUC= 0.77) requires validation in larger, independent cohorts. Future studies including both endometriosis patients and healthy controls are necessary to determine whether this ratio has any diagnostic or screening value beyond its potential role in distinguishing mild from severe disease.

Acknowledgement

The authors would like to acknowledge the use of artificial intelligence-based language assistance (ChatGPT, OpenAI) for improving the grammar, clarity, and language of the manuscript. The authors reviewed and approved the final version of the manuscript and take full responsibility for its content, interpretation, and conclusions.

Conflict of Interest

Authors declare no conflict of interest.

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